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CXCL12 N-terminal end is sufficient to induce chemotaxis and proliferation of neural stem/progenitor cells

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Abstract   Neural stem/progenitor cells (NSC) respond to injury after brain injuries secreting IL-1, IL-6, TNF-α, IL-4 and IL-10, as well as chemokine members of the CC and CXC ligand families. CXCL12 is one of the chemokines secreted at an injury site and is known to attract NSC-derived neuroblasts, cells that express CXCL12 receptor, CXCR4. Activation of CXCR4 by CXCL12 depends on two domains located at the N-terminal of the chemokine. In the present work we aimed to investigate if the N-terminal end of CXCL12, where CXCR4 binding and activation domains are located, was sufficient to induce NSC-derived neuroblast chemotaxis. Our data show that a synthetic peptide analogous to the first 21 amino acids of the N-terminal end of CXCL12, named PepC-C (KPVSLSYRCPCRFFESHIARA), is able to promote chemotaxis of neuroblasts *in vivo*, and stimulate chemotaxis and proliferation of CXCR4+ cells *in vitro*, without affecting NSC fate. We also show that PepC-C upregulates CXCL12 expression *in vivo* and *in vitro*. We suggest the N-terminal end of CXCL12 is responsible for a positive feedback loop to maintain a gradient of CXCL12 that attracts neuroblasts from the subventricular zone into an injury site.
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Introduction

CXCL12−CXCR4 axis plays important roles during CNS development, controlling cerebellar neuronal progenitor cells (NPC) migration (Ma et al., 1998; Zou et al., 1998), and increasing cerebellar and cortical NPC proliferation (Klein et al., 2001; Wu et al., 2009). In the mature brain, CXCL12 regulates neural stem/progenitor cell (NSC) occupancy of the subventricular zone (SVZ) vascular niche, and upregulates EGFR and integrin α6 expression stimulating the exit and migration of NSC-derived neuroblasts from the SVZ to the olfactory bulb (Kokovay et al., 2010). Involvement of CXCL12 and CXCR4 in brain pathologies has also been described. Migration of NSC towards a brain tumor expressing CXCL12 is mediated by the activation of CXCR4 (van der Meulen et al., 2009). Stroke and traumatic injuries to the CNS lead to an inflammatory response that includes local secretion of several chemokines that, among other effects, attract NSC-derived neuroblasts from the SVZ (Robin et al., 2006; Takeuchi et al., 2007; Itoh et al., 2009; Galindo et al., 2011; Jaerve and Müller, 2012).

CXCL12 binds and activates two G-protein coupled receptors, CXCR4 and CXCR7. Migration and proliferation of NSC and NPC are regulated *via* activation of CXCR4, whereas the activation of CXCR7 leads to cell survival (Bakondi et al., 2011). To activate CXCR4, CXCL12 binding domain formed by the amino acid residues 12−17 (RFFESH) binds to CXCR4 acting as an initial docking site for CXCL12. Following binding, the activation domain composed of amino acid residues 1−8 (KPVSLSYR) binds to the N-terminal end of the receptor, inducing a conformational change that, in turn, allows binding of G-protein to the intracellular domains of the receptor, leading to activation of signaling pathways involved in the control of migration, proliferation and survival (Crump et al., 1997; Huang et al., 2003; Saini et al., 2011).

Due to CXCL12 pleiotropic effects there is increasing interest to investigate this chemokine as a target for therapeutic strategies to treat a number of brain pathologies. CXCL12 pretreatment preserved dendritic spines of hippocampus neurons submitted to neurotoxicity induced by amyloid-β peptide oligomers *in vivo* (Raman et al., 2011). Increase in CXCL12 expression by astrocytes after brain ischemia attracts transplanted human umbilical cord cells expressing CXCR4 to the injury site. Inhibition of CXCL12 activity by neutralizing antibodies *in vivo* resulted in significant reduction in cord blood cells at the injured area, reinforcing the view that CXCL12−CXCR4 axis activates pathways that are keys for homing of CXCR4 expressing cells (Rosenkranz et al., 2010).

The use of CXCL12 to increase chemotaxis and improve survival of NSC to spinal cord and traumatic brain injuries (SCI, TBI) is an approach that has been explored by several groups, but there are still many molecular and cellular issues to be addressed. Here, we provide evidence that a synthetic peptide analogous to the N-terminal portion of CXCL12 containing the receptor binding and activating domains enhances chemotaxis of neuroblasts from the SVZ to an injured area in the cortex. Moreover, this effect seems to be mediated by upregulation of CXCL12 locally, possibly by astrocytes, as indicated by our *in vitro* experiments. The synthetic peptide is also able to promote NSC proliferation *in vitro*, without affecting differentiation. A modified peptide, in which we substituted cysteines 9 and 11 by alanines, was not chemotactic for CXCR4 expressing cells but reduced GFAP expression, suggesting that it could be useful to reduce astrocyte reaction *in vivo*. The data we present here suggest that the approach of using synthetic peptides that mimic CXCL12 chemotactic effect is relevant for the development of new therapeutic strategies to treat TBI and add information regarding the role of CXCL12 N-terminal end on its different biological activities.

Material and methods

Peptide synthesis

Peptides analogous to CXCL12 N-terminal were synthesized by Fmoc (9-fluorenilmetoxicarbonil) technique (Hirata et al., 1994) and characterized by HPLC (Shimadzu,Tokyo, Japan) using a reverse phase Econosil C18 column (5 μm; 4.6 × 150 mm). The peptides were purified by semi preparative HPLC using a reverse phase Econosil C18 column (5 μm; 22.5 × 250 mm), and were analyzed by MALDI-TOF using a TofSpec E Mass Spectrometer (Micromass, Manchester, UK). The synthetic peptides were diluted in 0.1 M PBS pH 7.4 at a final concentration of 3 M and maintained at −20 °C. The six peptides synthetized and used in this study had the following sequences:

Pep1:	KPVSLSYR-NH2
Pep2:	KPVSLSYR-OH
Pep3:	RFFESHIARA
Pep4:	KPVSLSYR-epsilon-aminocaproic acid-RFFESHIARA
PepA-A:	KPVSLSYRAPARFFESH
PepC-C:	KPVSLSYRCPCRFFESHIARA

Mice

C57Bl/6 mice used in this study were maintained in ventilated microisolator cages, under a cycle of light/dark (12 h/12 h), with free access to water and food. All surgical interventions, experimental protocols and handling of the animals followed international guidelines (http://www.iclas.org/harmonization. htm) and were approved by the Committee for Ethics in Research of Universidade Federal de São Paulo (CEP 0096/09).

Isolation of adult NSC and neurosphere formation

Adult NSC were obtained from 6 weeks old C57Bl/6 mice SVZ. Briefly, immediately after euthanasia, the brain was removed from skull, the SVZ was dissected and cells were dissociated by incubation with 125 mg/ml papain (Worthington Biomedical, Lakewood, USA), during 5 min at 37 °C. After mechanical dissociation, cells were strained through a sterile mesh (0.4 μm) (BD Biosciences, Franklin Lakes, USA) and plated on a 24-well plate at a density of 2 × 10^6 cells/well in PolyHema (Sigma-Aldrich, St Louis, USA) precoated flasks. Cells were cultured in DMEM high glucose:F12, 7:3 v/v (Gibco, San Francisco, USA), containing 2% B27 (Invitrogen, Carlsbad, USA), 20 ng/ml EGF (Sigma), 20 ng/ml FGF2 (R&D Systems, Minneapolis, EUA), 1% penicillin/streptomycin (Gibco), and 5 μg/ml heparin (Sigma). Neurosphere formation takes up to

21 days to occur, and during that time culture medium was changed every 4–5 days by centrifugation for 5 min at 358 ×g. Half of the volume of the conditioned medium was replaced by fresh medium.

Isolation of cerebellar NPC

Cerebellar NPC were isolated from neonatal mouse cerebellum as previously described (Klein et al., 2001; Zhou et al., 2007). The cell suspension containing NPC was platted on poly-L-lysine (Sigma) precoated plates at a density of 1×10^6 cells/35 mm dish or 3×10^5 cells/well on 24-well plates. Cells were maintained in DMEM/F12 1:1, containing 20 mM KCl, 36 mM glucose and penicillin/streptomycin, at 37 °C, 5% CO_2.

Astrocyte primary culture

Astrocytes were obtained from the forebrains of neonatal mice (P2). Briefly, mice were decapitated, the forebrains were removed aseptically from the skulls and meninges were excised carefully. After tissue mechanical dissociation, the suspension was strained through a sterile mesh (0.4 μm) and cells were seeded into culture flasks in DMEM low glucose, containing 10% fetal bovine serum (FBS, Cultilab, Campinas, Brazil), 1% glutamine 0.2 M and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 48 h in culture, the mixed glia was vigorously shaked, and this resulted in detachment of microglia, whereas astrocytes remained attached to the culture flask. Culture medium was renewed every 3–4 days, and astrocytes were characterized by GFAP immunocytochemistry.

Chemotaxis assay

Lymphocytes

Chemotaxis of lymphocytes was measured using the Boyden chamber assay. Adult human lymphocytes were obtained from peripheral blood of healthy volunteers and separated by Ficoll–Paque™ Plus (GE Healthcare, Piscataway, USA) gradient. Lymphocytes (2×10^5 cells/250 μl/chamber) were placed on the upper chamber, over a 5 mm pore membrane (Neuroprobe, Pleasanton, USA). The six synthetic peptides were diluted in RPMI (Invitrogen) and added to the bottom chamber at different concentrations. Cells were allowed to migrate overnight, and cells that went through the membrane were manually counted using a Neubauer chamber.

NSC

Chemotaxis of adult NSC was measured using a custom made Zigmond chamber (Zigmond and Hirsch, 1973). NSC cultured as neurospheres were plated at the center of the chamber and 3×10^{-8} M PepC-C was placed in one of the slots and DMEM was placed on the other slot in order to create a gradient surrounding the neurospheres. Cells were allowed to migrate overnight at 37 °C in a CO_2 incubator. After the incubation period, cells were photographed using an inverted microscope (Zeiss LSM510, Heidelberg, Germany) and counted.

TBI model, injection of peptides and tissue preparation

Adult C57Bl/6 mice received four intraperitoneal (i.p.) injections of BrdU (50 mg/kg body weight, in 0.1 M PBS pH 7.4), in two consecutive days (2 injections/day). Twenty four hours after the last injection, injury was performed as described next. All surgeries were performed under anesthesia with i.p. administration of ketamine chlorhydrate (66 mg/kg) (Dopalen Vetbrands, Brazil). Injury to the motor cortex was carried using a previously described protocol (Chiba et al., 2004; Coulson-Thomas et al., 2008) to produce a TBI. Briefly, a metal needle was chilled by immersing it on isopentane on dry ice and inserted four times into mice motor cortex (stereotaxic coordinates from bregma: AP +0.198 mm; LAT +0.175 mm; DV −0.15 mm). Immediately after the lesion, while the animals were still under anesthesia and using the same incision, peptides or vehicle (PBS) were injected at the injury site. Twenty four hours later, mice were anesthetized by an i.p. injection of sodium thiopental (Tiopentax, Cristália, São Paulo, Brazil) and euthanized. For qPCR experiments, the brain was removed from the skull, the motor cortex was dissected, and RNA was extracted by Trizol® (Invitrogen). Alternatively, mice were intracardially perfused with 4% paraformaldehyde (PFA) in 0.1 M PBS. The brain was removed from the skull and immersed in 4% PFA for 24 h at 4 °C, placed in 30% sucrose in PBS for 24 h at 4 °C, embedded in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, USA) and frozen using isopentane on dry ice. The embedded tissue was sliced into 12 μm coronal sections using a cryostat (Leica, model CM 1900, Wetzlar, Germany). The tissue slices were collected on silanized slides (Superfrost slides, Fisher Scientific, Philadelphia, USA) for immunofluorescence staining.

ELISA

Quantitation of CXCL12 in the supernatants of astrocyte cultures was performed following 24 h of experimental treatment. CXCL12 (PeproTech, São Paulo, Brazil) was added to the cultures at a final concentration of 100 ng/ml. CXCL12 DuoSet ELISA for mouse CXCL12 (R&D) was performed according to manufacturer specifications. ELISA was carried out in duplicates of samples from two independent experiments. CXCR4 was inhibited by AMD3465 (Tocris, Bristol, United Kingdom).

Quantitative real time PCR (qPCR)

Total RNA from dissected motor cortex or neurospheres was extracted by Trizol® (Invitrogen) and quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, EUA). Two micrograms of total RNA was reverse-transcribed with Oligo(dT)15 Primer and ImProm-II Reverse Transcription System with Recombinant RNasin® Ribonuclease inhibitor (Promega, Madison, USA) and $MgCl_2$. The primers used were: Cxcl12: sense 5′-ATC CTC AAC ACT CCA AAC TGT GCC-3′; antisense 5′-TTC AGA CCT AGG CTC CTC CTG TAA-3′; Cxcr4: sense 5′-AGC AGG TAG CAG TGA AAC CTC TGA-3′; antisense 5′-TGG TGG GCA GGA AGATCC TAT TGA-3′; Gfap: sense 5′-CTC AGT ACG AGG CAG TGG CC-3′; antisense 5′-CGG GAA GCA ACG TCT GTG A-3′; Nestin: sense 5′-AGC AAC TGG CAC ACC TCA AG-3′; antisense 5′-GGT GTC TGC AAG CGA AAG TTC-3′; Sox2: sense 5′-ATC CCA TCC AAA

TTA ACG CA-3′; antisense 5′-GAA GCG CCT AAC GTA CCA CT-3′; Cd133: sense 5′-TGG CAT TCT GTG TGG CTA TGT TGC-3′; antisense 5′-TGC GAT AGT ACT TGG CCA GCT TG-3′; Pax6: sense 5′-GAG GTC AGG CTT CGC TAA TG-3′; antisense 5′-GAG AGT TTT CTC CAC GGA CG-3′; Hprt: sense 5′-CTC ATG GAC TGA TTA TGG ACA GGA C-3′; antisense 5′-GCA GGT CAG CAA AGA ACT TAT AGC C-3′. qPCR was performed using SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, USA) and Mx3005P Real-Time PCR System (Agilent Technologies). The thermal cycling conditions were 10 min at 95 °C, and 40 cycles of 30 s at 95 °C, 1 min at 56 °C and 1 min at 72 °C. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Preparation of neurosphere slices for BrdU staining

Whole neurospheres were taken from the flasks and fixed in 4% PFA for 1 h at room temperature (r.t.). Neurospheres were washed three times with PBS and then transferred to PBS/10% sucrose solution for 2 h at r.t., PBS/20% sucrose solution for 2 h at r.t. and, finally, PBS/30% sucrose solution at 4 °C overnight. Neurospheres were then embedded in Tissue Freezing Medium and frozen using isopentane on dry ice. Spheres were sliced at 10 μm on a cryostat and placed on silanized slides.

Proliferation assays

[3H]-Thymidine incorporation

Cerebellar NPC proliferation was measured basically as previously described (Porcionatto et al., 1998). Briefly, NPC were obtained from P6 mice cerebella and were incubated in the presence or absence of 10% FBS and peptides. [3H]-Thymidine (0.5 μCi/ml) was added and cells were incubated for 24 h. After the incubation period the amount of incorporated [3H]-thymidine was measured by liquid scintillation counting.

BrdU incorporation and detection

Adult NSC proliferation *in vitro* was measured by immunocytochemistry and flow cytometry using Fluorescence Activated Cell Analyser (Beckman Coulter, Fc500, Fullerton, USA) using Cell Quest (The Cell Quest™ Software, Becton Dickinson Immunocytometry Systems, San Jose, USA), after BrdU incorporation. Adult NSC were cultured as neurospheres and maintained in the presence or absence of peptides during 4 days at 37 °C in a CO_2 incubator. BrdU (0.2 μM) was added to the medium in the last 24 h and, after this incubation period, was analyzed either by immunofluorescence or by flow cytometry. For flow cytometry, neurospheres were dissociated with trypsin to yield a single cell suspension, and then were fixed by 4% PFA in 0.1 M PBS, incubated in HCl 1.5 M for 30 min at 37 °C, washed three times 10 min each in PBS and blocked in 5% FBS and 0.1% Triton X-100 (AMRESCO, Solon, USA) in PBS. For immunofluorescence, the slices were incubated with anti-BrdU (rat IgG, 1:300; Axyll/Accurate Chemical & Scientific Corporation, North Hempstead, USA) for 2 h and washed three times in PBS. Cells were incubated with Alexa Fluor488-conjugated goat anti-rat IgG, 1:300 (Molecular Probes, Carlsbad, USA) for 1 h and washed three times in PBS. DAPI solution (1:1000, Molecular Probes) was used as a nuclear stain.

Differentiation assay

Adult NSC were cultured as neurospheres and maintained for 4 days in the presence or absence of pepA-A and PepC-C. Expression of neural and stem cell markers was assessed by flow cytometry using anti-GFAP (DAKO, Glostrup, Denmark), anti-β-III tubulin (Sigma), anti-Nestin (Millipore, Temecula, USA) and anti-NeuN (Millipore). Cells were incubated with 1:300 dilutions of primary antibodies for 1 h. Cells were washed with PBS, incubated with a 1:300 dilution of the secondary antibodies (anti-rabbit Alexa 546-conjugated [GFAP], anti-mouse Alexa 555-conjugated [β-III tubulin], anti-mouse Alexa 350-conjugated [Nestin], anti-rat Alexa 488-conjugated [NeuN], Invitrogen). Ten-thousand events were acquired per sample with fluorescence measured in logarithmic scales using flow cytometer Fluorescence Activated Cell Analyser (Beckman Coulter) and Cell Quest (The Cell Quest™ Software). Forward and side light-scatter gates were used to exclude dead cells and debris. Data were gated using side scatter (linear scale) and fluorescence signals (logarithmic scale). Negative controls were carried out in the absence of primary or secondary antibodies. Three experiments were carried out in duplicates.

Immunofluorescence

Fixed coronal brain sections or the neurospheres were exposed to 0.1% Triton X-100 for 10 min, washed in PBS three times, blocked with 5% FBS for 1 h at r.t. and then incubated overnight at 4 °C with the primary antibodies rabbit anti-GFAP, mouse anti-Nestin, mouse anti- anti-β-III tubulin, mouse anti-NeuN and rat anti-BrdU with 1:300 dilutions. The motor cortex sections or the neurospheres were then washed in PBS and incubated with appropriate secondary antibodies (Alexa Fluor546-conjugated [GFAP], goat anti-mouse IgG 1:300, AlexaFluor488-conjugated [NeuN] goat anti-rabbit 1:300, Alexa Fluor488 [BrdU] anti-rat IgG, 1:300, and anti-mouse Alexa 555-conjugated [β-III tubulin] Invitrogen). Nuclei were stained with DAPI (1:1000). After immuno- and DAPI staining, glass slides were mounted using Fluoromount G (Electron Microscopy Sciences, Hatfield, USA). The fluorescently labeled cells were analyzed using a Zeiss LSM510 scanning confocal inverted microscope (Zeiss, Oberkochen, Baden-Wurttemberg, Germany) and co-localization images were generated using Zeiss LSM Image Browser. In order to quantify BrdU + cells at the injury site, two consecutive sections of motor cortex stained for GFAP (green) and BrdU (red) were photographed and photos were transferred to Adobe Photoshop®. The total number of BrdU + cells was counted blind and the result is the average of three animals.

Statistical analysis

Statistically significant differences were evaluated by one-way ANOVA followed by Tukey post-test using the GraphPad Prism software version 5.01 (GraphPad Software, USA). Results are expressed as mean ± SEM and were considered significant if $p < 0.05$.

Results

CXCL12 N-terminal synthetic peptides promote migration of CXCR4 expressing cells

We synthesized six peptides analogous to CXCL12 N-terminal end to use in this study. Two of these peptides were composed of the first 8 amino acids of CXCL12, mimicking the region that contains the receptor activation domain (Pep1: KPVSLSYR-NH$_2$; Pep2: KPVSLSYR-OH); one peptide had the sequence of 12−21 amino acids, that corresponds to the receptor binding domain (Pep3: RFFESHIARA); and three peptides carried both, the receptor binding and receptor activating domains. The difference among these three peptides was that one of them had the exact structure of the first 21 amino acids of the native CXCL12 N-terminal end (PepC-C: KPVSLSYRCPCRFFESHIARA); the second had the two cysteines that are critical for CXC chemokine activity (C9 and C11) substituted by alanines (PepA-A: KPVSLSYRAPARFFESH), and the third had the CXC tripeptide substituted by ε-aminocaproic acid, that functions as a linker to separate the binding and activation domains (Pep4: KPVSLSYR-ε-aminocaproic acid-RFFESHIARA). Chemotactic activity of the peptides was first tested using lymphocytes, cells that express CXCR4. The use of blind end chambers (Boyden chamber) to test chemotaxis of lymphocytes towards a source of chemokines, such as CXCL12, has been extensively described in the literature. In this assay, concentrations of CXCL12 varying from 0.5 to 1.0×10^{-7} M are able to promote lymphocyte migration (Pelletier et al., 2000). The ability of each synthetic peptide to promote chemotaxis of lymphocytes in a Boyden chamber assay was tested in concentrations varying from 1.0×10^{-8} M to 1.0×10^{-4} M. Among the six peptides, PepC-C showed the highest activity, stimulating the migration of around 70% of the lymphocytes added to the upper chamber at a concentration of 3.0×10^{-8} M (Fig. 1A). Peptides with activation and binding domains only, Pep3 and Pep1, respectively, showed lower chemotactic activity (~30−40% of cells migrated) even when used at concentration 10 times higher than the effective concentration found for PepC-C. Substitution of C9 and C11 by alanines abolished the chemotactic activity of the peptide, and PepA-A only showed some chemotactic activity (~20% of cells migrated) when used at a concentration 100 times higher than the concentration found to be effective for PepC-C. From the six peptides tested, only Pep4 did not promote chemotaxis of lymphocytes even at a concentration 1000 times higher than the effective concentration encountered for PepC-C (Fig. 1A).

Based on the lymphocyte chemotaxis assay, PepC-C was selected to test the ability of CXCL12 N-terminal to promote migration, proliferation and differentiation of adult SVZ NSC-derived neuroblasts. PepA-A was selected to be used as control peptide because it presented low chemotaxis activity and differed from PepC-C by only two aminoacids (C9A and C11A). Before looking at proliferation and expression of cell differentiation markers we tested the peptides as promoters of NSC migration. NSC from the SVZ form floating neurospheres in culture and, if allowed to adhere to laminin, NSC-derived cells (glia and neuroblasts) migrate out of the sphere, recapitulating the exit of type A cells (neuroblasts) from the neurogenic niche

(Alvarez-Buylla & Lim, 2004). PepC-C and PepA-A were used in concentrations which stimulated the highest percentage of lymphocyte migration, PepC-C was used at 3×10^{-8} M and PepA-A at 3×10^{-6} M, respectively. Only PepC-C was able to increase migration of cells out of the neurospheres when added uniformly to the culture medium (Fig. 1B). A gradient of PepC-C also promoted migration of cells out of the neurospheres on a Zigmond chamber assay, demonstrating that the peptide not only stimulates random migration but also has chemotactic activity (Fig. 1B). When submitted to a uniform concentration of peptides, cells migrated longer distances from the neurosphere when compared to untreated cells, similar to what we had observed for cells treated with CXCL12 (Fig. 1C). In addition, the percentage of neurospheres that had cells migrating more than 150 μm was higher when cells were treated either with CXCL12 or PepC-C when compared to cells treated with PepA-A or not treated (Fig. 1D).

CXCL12 N-terminal peptide induces chemotaxis of neuroblasts *in vivo*

As the synthetic peptides were able to stimulate migration and chemotaxis of cells *in vitro*, we asked if this activity could also be observed *in vivo*. Using an animal model for TBI, we evaluated the exit of neuroblasts from the SVZ 24 h after an injury was produced in the motor cortex of mice. Neuroblasts are constantly generated by NSC in the SVZ neurogenic niche, so they can be labeled with BrdU and traced afterwards. As expected, in non-injured brains we observed BrdU + cells lining the lateral ventricle wall (Fig. 2A), and when the injury was produced in the motor cortex, BrdU + cells could be seen leaving the SVZ (Fig. 2B). Injection of PepC-C, but not PepA-A, at the injury site immediately after injury, greatly increased the amount of BrdU + cells in the migratory stream (Figs. 2A-D). Quantification of BrdU + cells at the injury site revealed that PepC-C more than doubled the number of cells that arrived at the injury site in 24 h when compared to untreated animals, treated with vehicle or treated with PepA-A (Fig. 3).

Although we did not measure the peptides half-life *in vivo*, it is expected that they would be short living. It is likely that the peptides probably last only a few minutes after being injected *in situ* due to the presence of several proteases at the injury site. To explain how a single injection of peptide would enhance attraction of neuroblasts to the injury site we looked at the expression of CXCL12 locally and observed that PepC-C, but not PepA-A, was able to triple the levels of Cxcl12 mRNA locally, after 24 h (Fig. 4A), without interfering with the expression of Cxcr4 (Fig. 4B).

Astrocytes respond to injury signals secreting CXCL12, among other soluble factors and molecules associated with the extracellular matrix. In order to investigate whether PepC-C was able to stimulate CXCL12 expression and secretion by astrocytes we treated cultures of mice astrocytes with PepC-C and PepA-A, and measured the concentration of CXCL12 in the culture medium. PepC-C and PepA-A, alone or combined, did not increase the release of CXCL12 to the culture medium (Fig. 4C). Next, we asked if PepC-C could stimulate a positive feedback loop of CXCL12 secretion. To investigate this, CXCL12 was added to the astrocytes in culture to mimic CXCL12 present
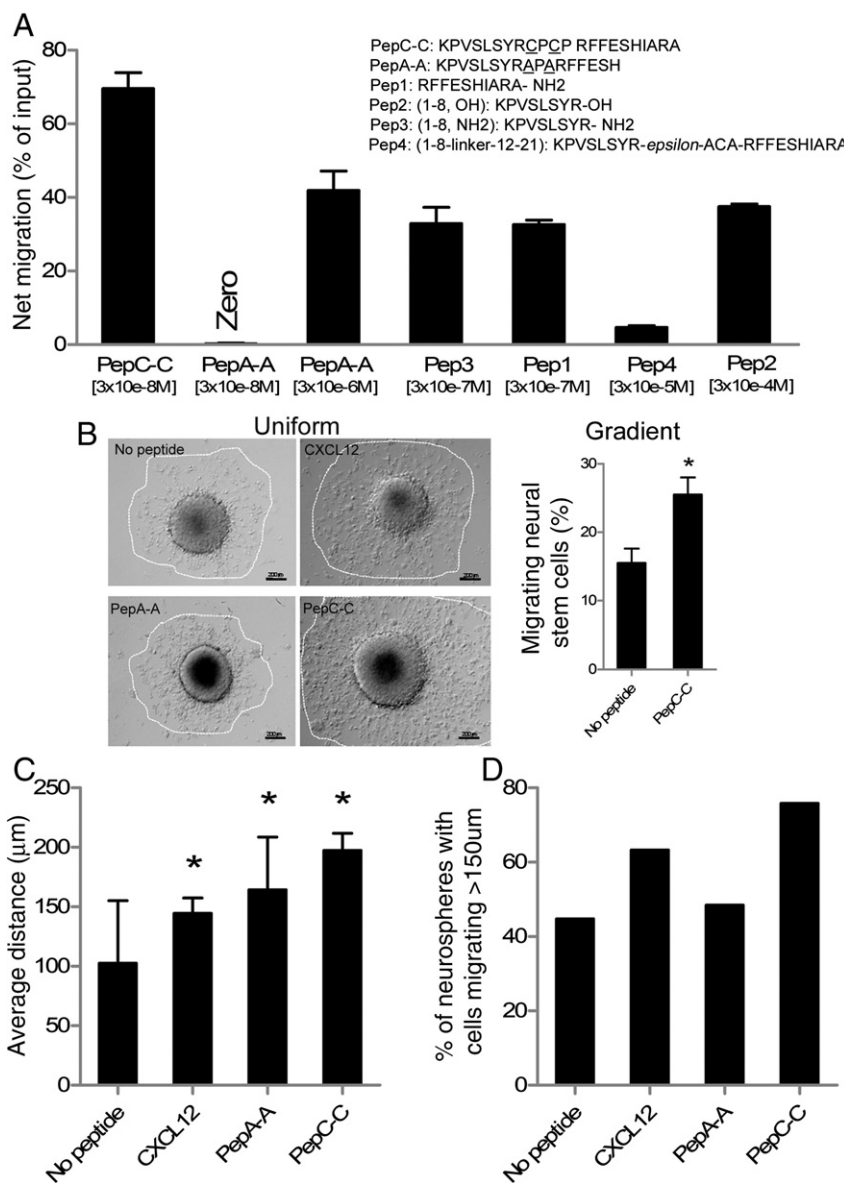
Figure 1 Synthetic peptides analogous to CXCL12 N-terminal are chemotactic for CXCR4 expressing cells. (A) Lymphocyte chemotaxis in response to the concentration of the synthetic peptide that showed highest stimulation. The number of lymphocytes that migrated in the Boyden chamber assay was calculated by counting cells that migrated to the bottom in 24 h in relation to the initial number of cells placed on the upper chamber (initial input 2×10^5 cells). (B) NSC migration and chemotaxis assays were performed by adding CXCL12, peptides PepA-A and PepC-C to neurosphere culture medium (Uniform), or performed in a Zigmond chamber (Gradient). The distance traveled by migrating cells from the neurospheres is highlighted by the white lines. For Zigmond chamber assay, PepC-C was added to one slot of the chamber and culture medium was added to the other slot. Neurospheres were placed between the two slots and cells that migrated towards each slot were counted. (C) Distance traveled by NSC was calculated by measuring the distance from the border of the neurospheres to the border of the migration radius (white lines in panel B), using ImageJ. CXCL12, PepA-A and PepC-C treatments were compared to "no peptide" (* $p < 0.05$). (D) Percentage of neurospheres that had cells migrating 150 μm from the neurospheres border or more, after 18 h of treatment (* $p < 0.05$). Number of neurospheres analysed for each treatment condition: no peptide, 29; CXCL12, 30; PepA-A, 31; and PepC-C, 29.

at the injury site *in vivo*. CXCL12 secreted to the medium was measured, and we observed that the addition of PepC-C increased CXCL12 concentration by three fold, whereas PepA-A did not (Fig. 4C). Inhibition of CXCL12 receptor (CXCR4) did not interfere with the increase in CXCL12 secretion stimulated by Pep-C-C, indicating that CXCL12 production and secretion is independent of CXCR4 activation.

PepA-A represses expression of Gfap at the injury site

As part of the glial reaction, injury to the brain causes upregulation of Gfap and Nestin. In the model used in this study we observed increased expression of Gfap and Nestin
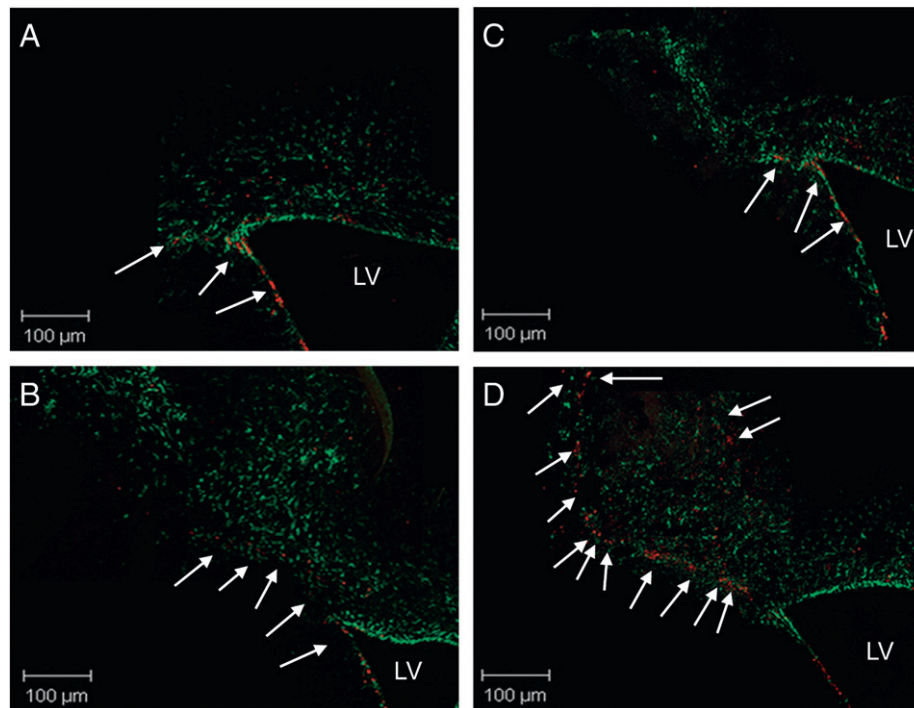
Figure 2   PepC-C stimulates neuroblast migration *in vivo*. Sections of motor cortex showing BrdU + neuroblasts (red) and GFAP + astrocytes (green). (A) Control, not injured; (B) injured, vehicle injected; (C) injured, PepA-A injected; (D) injured, PepC-C injected. To prelabel neuroblasts, mice received four i.p. injections of BrdU, in two consecutive days (two injections/day); 24 h after the last BrdU injection, injury was performed. Peptide injections were made at the injury site immediately after injury. Animals were euthanized 24 h after injury. Arrows indicate BrdU + neuroblasts, and the injury site is in the upper part of each photo. PepC-C increased the number of cells that left LV towards injury site 24 h after injury. LV: lateral ventricle.

24 h after injury (Fig. 5A). Administration of PepA-A downregulated the expression of both genes to levels similar to control (non injured brain). In contrast, PepC-C had no effect on the expression of Gfap and Nestin, but upregulated the expression of the proliferation marker Sox2, which was unaffected by injury or by treatment with PepA-A (Fig. 5A).

PepA-A also downregulated the expression of Gfap by NSC *in vitro* without affecting Nestin and Sox2 expression (Fig. 5B). Similar to what we had observed *in vivo*, PepC-C did not change Gfap expression by NSC when compared to control untreated cultures, and upregulated Sox2. Nestin expression *in vitro* was upregulated by PepC-C and was not affected by treatment with PepA-A (Fig. 5B).

CXCL12 N-terminal is sufficient to induce neural stem cell proliferation but does not change NSC fate

Because PepC-C upregulated the expression of Sox2 both *in vitro* and *in vivo*, we decided to evaluate its effect on neuronal precursor proliferation. Neurospheres treated with CXCL12 or PepC-C showed increased mRNA expression of the stem cell marker Cd133, when compared to non-treated or PepA-A treated cultures (Fig. 6A). Using cerebellar NPC, cells that express CXCR4, we observed increased proliferation when cells were treated with PepC-C, even in the absence of FBS, whereas PepA-A did not show any proliferative activity (Fig. 6B).

NSC cultured in the presence of PepC-C showed increased proliferation assessed by BrdU incorporation and analyzed by immunocytochemistry and flow cytometry (Figs. 6C–D). TUNEL assay revealed that the peptides did not interfere with the apoptosis of NSC *in vitro* (Fig. 6E).

Following the observation that the peptides were able to stimulate migration, chemotaxis and proliferation of NSC, as well as to modify gene expression, we then evaluated if they could affect NSC fate. We first looked at the expression of Pax6, a transcription factor known to be critical for differentiation of neural precursors into neurons. The results show that PepA-A downregulated Pax6 in NSC when compared to untreated cells (Fig. 6 F). Cytometry analysis showed that the percentage of GFAP+, β-III tubulin+, Nestin + and NeuN + cells did not change with treatment with PepC-C or PepA-A (Fig. 6 F).

Discussion

In this study we provide evidence that the N-terminal end of CXCL12, a region containing CXCR4 binding and activation domains, is sufficient to induce both migration and proliferation of SVZ-born neuroblasts. Our experimental approach was to synthesize peptides that are analogous to CXCL12 N-terminal, introducing modifications such as replacement of the tripeptide C9-P10-C11 by a linker that separates the receptor binding and receptor activation domains or replacing C9 and C11 by A (PepA-A). As predicted, the synthetic peptides
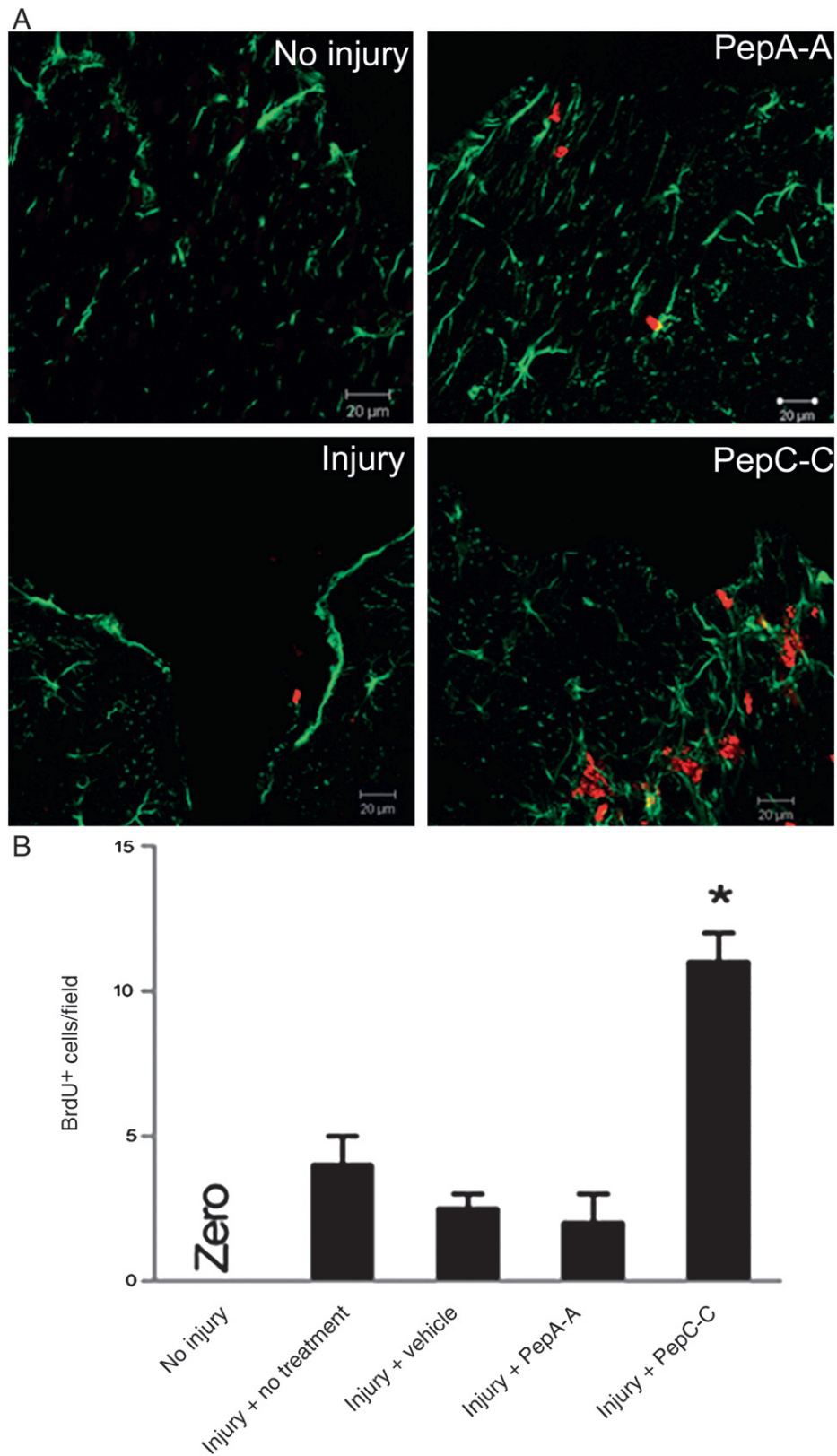
Figure 3   PepC-C increased the number of neuroblasts that migrated from the SVZ and arrived at the injury site. (A) Sections of motor cortex injury site, showing BrdU + neuroblasts (red) and GFAP + astrocytes (green). (B) Quantification of BrdU + cells at the injury site 24 h after injury. PepC-C increased the number of cells that arrived at the injury site when compared to injury not treated, treated with vehicle or treated with PepA-A (* $p < 0.05$).
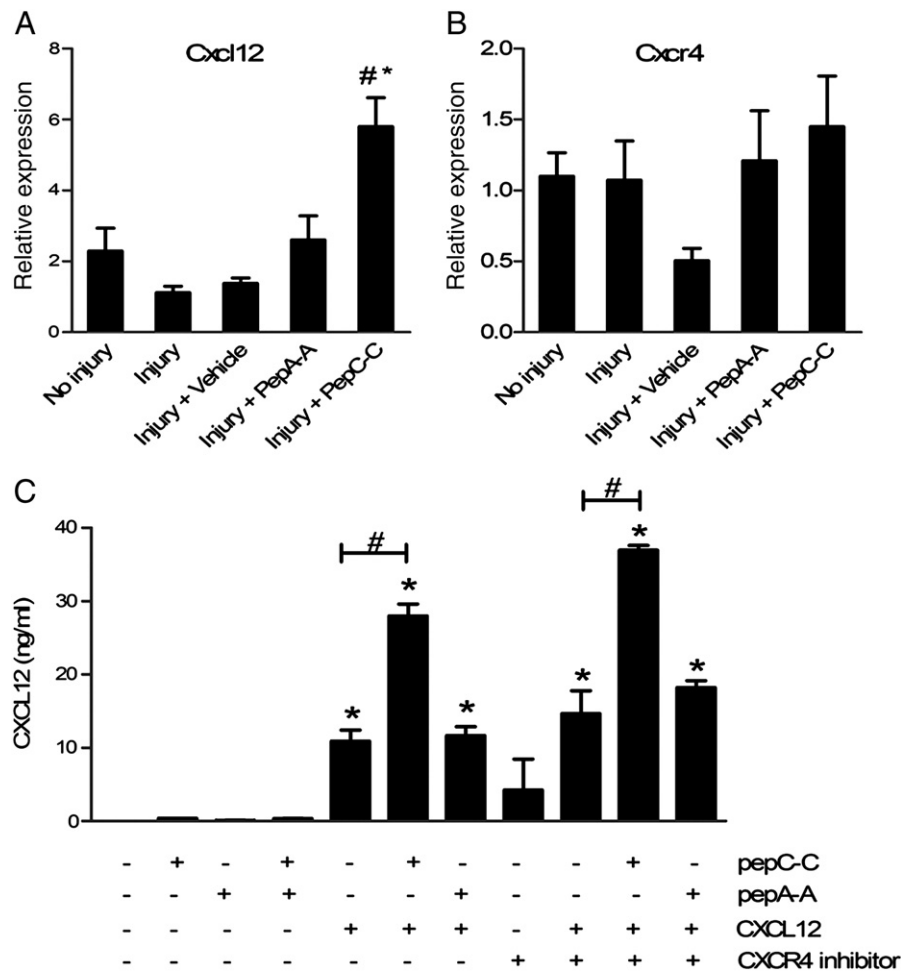
Figure 4 PepC-C stimulates expression of Cxcl12 *in vivo* and *in vitro*. RNA was extracted from primary motor cortex of mice and qPCR was used to quantify the expression of Cxcl12 (A) Cxcr4 (B). Results presented are average of mRNA from 3 animals, qPCR was performed in triplicates and values are expressed as mean ± SEM, and are relative to the expression of Hprt. After 24 h, PepC-C upregulated Cxcl12 expression locally, without interfering with Cxcr4 expression (* $p < 0.05$ when the treated group was compared to "No injury"; # $p < 0.05$ when the treated group was compared to "Injury"). (C) Astrocytes were cultured in the presence (+) or absence (−) of CXCL12 (100 ng/ml), PepA-A, PepC-C and CXCR4 inhibitor (AMD3465). The total amount of CXCL12 released from astrocytes during 24 h was measured by ELISA. PepC-C plus CXCL12 increased the amount of CXCL12 in the medium, and this response was not inhibited by AMD3465 (* $p < 0.05$ comparison of each treatment with no treatment; # $p < 0.05$).

bearing only the binding domain (Pep3) or the activation domain (Pep1 and Pep2) showed little chemotactic activity, and were less active when compared to the complete CXCL12 molecule or to the peptide that had both domains (PepC-C). Similar results were obtained by Crump et al. (1997), using chimeras containing CXCL12 N-terminal end (receptor activation domain) and the REFFESH sequence (receptor binding domain) and showed that they were chemotactic for lymphocytes, demonstrating the chimeras contained the contact residues essential for functional CXCL12. Here, we not only corroborate those findings but in addition demonstrate that the alanine-modified peptide (PepA-A) was less chemotactic than CXCL12 and PepC-C and had no proliferative activity on CXCR4+ NSC or cerebellar NPC *in vitro*. Once we determined that PepC-C was as good chemotactic factor as CXCL12 *in vitro* we evaluated its ability to promote chemotaxis *in vivo*.

It has been extensively described that after an injury to the brain or spinal cord the expression of CXCL12, among other chemokines and cytokines that are part of the inflammatory response, rises at the injury site. Following ischemic stroke, SCI or TBI, the blood−brain barrier becomes dysfunctional or disrupted, allowing circulating immune cells to infiltrate, interact with and activate resident immune cells. Activated glial cells (astrocytes, microglia and oligodendrocytes) start expressing chemokines such as CCL2, CCL3, CXCL1, CXCL2 and CXCL12, as part of the local inflammatory response (Kim et al., 1995; Takami et al., 1997; Hill et al., 2004; Pineau et al., 2010; Galindo et al., 2011). The current knowledge about the dynamics of chemokine expression in CNS injury and repair has been recently reviewed by Jaerve and Müller (2012).

Using a TBI mouse model we show here that PepC-C, but not PepA-A, is able to promote migration of NSC from the SVZ towards the injury site. Twenty four hours after injury it was possible to observe that the number of BrdU prelabeled cells arriving at the traumatic injury and peptide administration site doubled when compared to animals subjected to injury but not treated or treated with vehicle or A-substituted
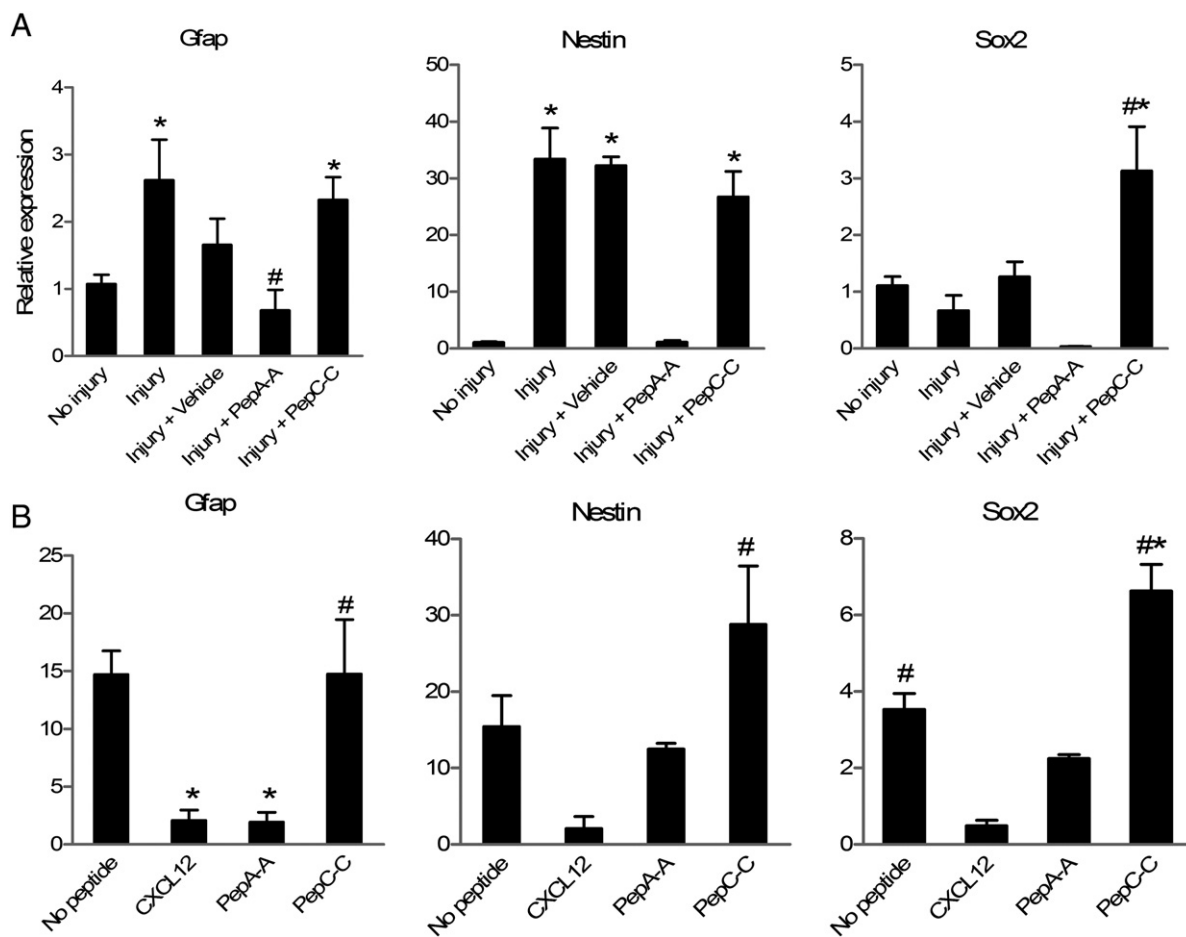
Figure 5   CXCL12 N-terminal peptides alter injury-related gene expression. Relative expression of Gfap, Nestin, Sox2 in motor cortex (A) and by neurospheres (B) in response to treatment with PepA-A, PepC-C and CXCL12. Results presented are average of mRNA from 3 animals (motor cortex) or 3 independent experiments (neurospheres); qPCR was performed in triplicates and values are expressed as mean ± SEM (* $p < 0.05$ when each treatment group was compared to "No injury" or "No peptide"; # $p < 0.05$ when each treatment group was compared to "Injury" or "CXCL12").

peptide (PepA-A). We have not performed experiments to measure the peptide half-lives at the site of injury and injection, but there is data suggesting that the peptides would not last long. For example, a tripeptide analogous to the N-terminal of insulin-like growth factor I (IGF-I) lasted only 3 h following intracerebroventricular administration in the presence of peptidase inhibitors (Baker et al., 2005). Based on that and on the fact that several proteases are secreted after injury to the CNS (Agrawal et al., 2008), we considered that the peptides would not last for 24 h of *in vivo* experiment. To find a possible mechanism for the prolonged duration of chemotactic activity of PepC-C *in vivo*, we hypothesized that a gradient of CXCL12 could be formed if PepC-C stimulated the release of high amounts of CXCL12 at the injury site that would diffuse away from the source, reaching the SVZ cells. To verify that, we looked at Cxcl12 mRNA at the injury site 24 h after injury and peptide administration and observed that PepC-C, but not PepA-A, doubled the local levels of Cxcl12 mRNA. There is no information regarding the modulation of CXCL12 expression by astrocytes after TBI, but based on data that shows upregulation of CXCL12 expression by astrocytes in response to ischemia (Hill et al., 2004; Miller et al., 2005), we

investigated if those cells were the ones responsible for the increase in Cxcl12 mRNA we observed *in vivo*. To do that, we exposed astrocytes in culture to the peptides and measured CXCL12 protein by ELISA. Surprisingly, PepC-C alone was not able to induce the release of CXCL12 to the culture medium, but acted synergistically with CXCL12, increasing the concentration of CXCL12 in the culture medium after 24 h. It is well documented that CXCL12 is internalized upon binding to CXCR4 (Amara et al., 1997; Signoret et al., 1997; Haribabu et al., 1997). When astrocytes were treated with CXCL12 alone the concentration of the chemokine in the culture medium after 24 h was ~10 ng/ml, that represents ~10% of the original input (100 ng/ml), so the increase from ~10 ng/ml to ~30 ng/ml when cells were exposed to CXCL12 + PepC-C could be explained by inhibition of CXCR4 internalization. To verify this we treated the astrocytes in the presence of a specific CXCR4 inhibitor (AMD3465) (Hatse et al., 2002) and did not observe any difference in the concentration of CXCL12 in the presence of peptides and/or CXCL12 and absence of AMD3465, indicating that PepC-C stimulated expression and secretion of CXCL12 rather than inhibited its internalization *via* CXCR4 activation. Two aspects are very interesting about
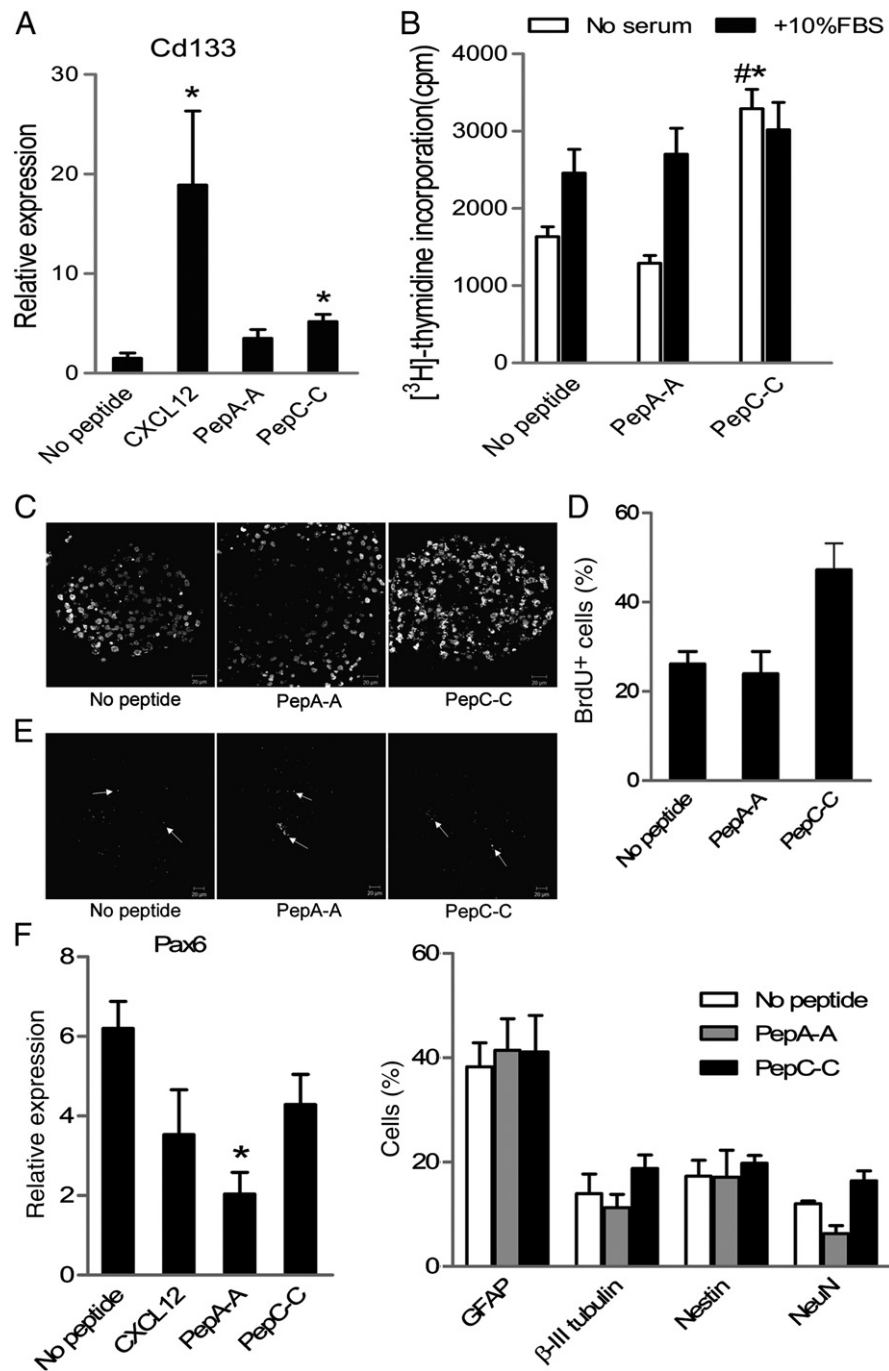
Figure 6 CXCL12 N-terminal is sufficient to increase proliferation of NSC but does not affect differentiation. (A) PepC-C upregulated the expression of the stem cell marker Cd133 by NSC cultured as neurospheres. (B) Proliferation of cerebellar NPC was assessed by [^{3}H]-thymidine incorporation in the presence or absence of peptides and 10% FBS. Proliferation of NSC was measured by BrdU incorporation using immunohistochemistry (C) and flow cytometry (D). (E) Arrows indicate nuclei labeled by TUNEL assay. (F) PepA-A downregulated the expression of Pax6 by NSC cultured as neurospheres. PepA-A and PepC-C did not alter differentiation of NSC, measured by GFAP, β-III tubulin, Nestin and NeuN using flow cytometry. qPCR was performed in triplicates and values are expressed as mean ± SEM. Results presented are average of 3 independent experiments. [^{3}H]-Thymidine incorporation results presented are the average of 3 independent experiments, each performed in duplicates (#$p < 0.05$ compared to "no serum/no peptide"; *$p < 0.05$ compared to "+10%FBS/no peptide").

these data. First, PepC-C alone cannot stimulate CXCL12 expression, it requires CXCL12 to be effective; and second, it does not involve CXCR4 signaling. Our suggestion is that this stimulatory effect could be elicited by the activation of CXCR7, a recently described G-protein coupled receptor for CXCL12 (Burns et al., 2006; Schönemeier et al., 2008; Odemis et al., 2010). The molecular mechanisms involved in PepC-C-induced CXCL12 expression/secretion by astrocytes

were not the focus of the present work and deserve further investigation.

Once we determined that PepC-C upregulated CXCL12 expression at the injury site and enhanced chemotaxis of NSC from the SVZ, we further investigated if the peptides would modulate the expression of markers for peri-injury reactive astrocytes Gfap, Nestin and Sox2 (Nakagomi et al., 2009; Shimada et al., 2010, 2012). Similar to what is observed for ischemia, Gfap and Nestin were upregulated by the traumatic injury, whereas Sox2 expression did not change. The expression of Gfap and Nestin after injury was not affected by the administration of PepC-C, but the expression of Sox2 was greatly increased by this treatment, suggesting that PepC-C could be increasing proliferation of astrocytes at the injury site. Upregulation of Sox2 was also observed *in vitro*, when adult NSC cultured as neurospheres were exposed to PepC-C, and we confirmed PepC-C proliferative effect on NSC and cerebellar NPC, *in vitro*, by [^{3}H]-thymidine and BrdU incorporation.

One unexpected set of data we got was the downregulation of Gfap by the modified peptide PepA-A both *in vivo* and *in vitro*. PepA-A has low chemotactic activity *in vitro*, no chemotactic activity *in vivo*, does not increase CXCL12 expression by astrocytes and does not have proliferative effect on NSC or cerebellar NPC *in vitro*. Besides downregulating expression of Gfap, the substitutions C9A and C11A led to downregulation of Pax6, a transcription factor involved in NSC fate determination. Pax6 induces neurogenic fate in SVZ glial progenitors by repressing Olig2 expression (Jang and Goldman, 2011) and the loss of Pax6 enhances astrogenesis (Sugimori et al., 2007; Sakayori et al., 2012). In conclusion, we show here that the N-terminal of CXCL12, a region containing the receptor binding and activation domains, is sufficient to stimulate proliferation and chemotaxis of NSC from the adult SVZ to an injury site in the cortex. These actions are likely performed *via* activation of the G-protein coupled receptor CXCR4, but we do not rule out the participation of CXCR7 in signaling a proposed positive feedback loop for CXCL12 expression and secretion.

Conflict of interest

All authors declare not to have any actual or potential conflict of interest including financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, this work.

Acknowledgments

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